

Pseudomonas aeruginosa May Accumulate Drug Resistance Mechanisms without Losing Its Ability To Cause Bloodstream Infections[▽]

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In this study, we systematically investigated the resistance mechanisms to β -lactams, aminoglycosides, and fluoroquinolones of 120 bacteremic strains of *Pseudomonas aeruginosa*. Pulsed-field gel electrophoresis genotyping showed that 97 of these strains were represented by a single isolate, 10 by 2 and 1 by 3 clonally related isolates, respectively. Seventy-five percent (90 out of 120) of the bacteremic *P. aeruginosa* strains displayed a significant resistance to one or more of the tested antimicrobials (up to 11 for 1 strain). These strains were found to harbor a great diversity of resistance mechanisms (up to 7 in 1 strain), leading to various levels of drug resistance. Interestingly, 11 and 36% of the isolates appeared to overproduce the MexAB-OprM and MexXY-OprM efflux systems, respectively. Altogether, our results show that *P. aeruginosa* may accumulate intrinsic (overproduction of cephalosporinase AmpC, increased drug efflux, fluoroquinolone target mutations, and deficient production of porin OprD) and exogenous (production of secondary β -lactamases and aminoglycoside-modifying enzymes) resistance mechanisms without losing its ability to generate severe bloodstream infections. Consequently, clinicians should be aware that multidrug-resistant *P. aeruginosa* may remain fully pathogenic.

Pseudomonas aeruginosa is a well-known nosocomial pathogen responsible for a wide range of mild to severe infections. Naturally resistant to many antimicrobial agents used in the hospital, this bacterium has the distinctive capacity via multiple mechanisms to become resistant to virtually all the antibiotics available commercially (12, 37). For example, significant resistance to β -lactams may arise from stable up-regulation of the intrinsic cephalosporinase AmpC, acquisition of transferable β -lactamases, increased drug efflux (MexAB-OprM, MexCD-OprJ, and MexXY-OprM systems), or outer membrane impermeability (alteration of porin OprD) (21, 22). Resistance to aminoglycosides may involve aminoglycoside- or 16S RNA-modifying enzymes, as well as the MexXY-OprM efflux pump (26). Similarly, fluoroquinolone resistance may be due to mutations in target genes (*gyrA*, *gyrB*, *parC*, and *parE*) or to drug efflux systems (MexAB-OprM, MexCD-OprJ, MexEF-OprN, and MexXY-OprM) (19, 27).

Despite an abundant literature, little is known about the prevalence of these different mechanisms among clinical strains of *P. aeruginosa*, how they contribute to drug resistance

levels (see reference 7 for a recent review), and whether they influence bacterial virulence. For instance, recent data on in vitro mutants have suggested that the virulence of *P. aeruginosa* might be reduced when the Mex efflux systems are overexpressed (11, 13, 20, 29, 32).

In this study, we demonstrate that *P. aeruginosa* may accumulate numerous mechanisms of resistance to β -lactams, aminoglycosides, and fluoroquinolones while involved in bloodstream infections.

MATERIALS AND METHODS

Bacterial strains. One hundred twenty strains of *P. aeruginosa* corresponding to the initial isolates of distinct bacteremic episodes were prospectively collected between 1999 and 2004 in six French teaching hospitals that are members of the GESPA group (Groupe d'Etude des Septicémies à *Pseudomonas aeruginosa*). The genotypic relatedness of these isolates was investigated by pulsed-field gel electrophoresis (CHEF-DR III; Bio-Rad, Ivry sur Seine, France) of *DraI*-macro-restricted genomic DNA as described previously (31). According to consensual guidelines (33), two isolates were considered clonally related when their pulsed-field gel electrophoresis profiles exhibited less than three bands of difference. The well-characterized mutants PT629 and MutGR-1 from wild-type reference strain PAO1 were used as positive controls in reverse transcription-PCR (RT-PCR) experiments for the identification of gain-of-efflux mutants overexpressing pumps MexAB-OprM and MexXY-OprM, respectively (23).

Drug susceptibility testing. The MICs of selected antibiotics were determined with the conventional macrodilution technique in Mueller-Hinton agar with calibrated concentrations of divalent cations (MHA; BBL, Cockeysville, MD) (2). Strains were classified as "susceptible," "intermediate," or "resistant" according to the CLSI breakpoints (25).

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TABLE 1. Susceptibility rates of the 120 bacteremic isolates of *P. aeruginosa*

Antibiotic	MIC range ($\mu\text{g/ml}$)	Susceptibility rate (%) ^a			MIC ₅₀ ($\mu\text{g/ml}$)	MIC ₉₀ ($\mu\text{g/ml}$)
		S	I	R		
Piperacillin-tazobactam ^b	<0.5–256	95.0		5.0	4	64
Ceftazidime	0.5–64	90.0	6.7	3.3	2	8
Imipenem	0.5–64	82.5	6.7	10.8	2	16
Cefepime	1–64	81.7	15.0	3.3	4	16
Aztreonam	<0.5–64	75.8	17.5	6.7	4	16
Ticarcillin	4–>256	77.5	11.7	10.8	32	256
Amikacin	\leq 0.5–128	93.3	5.0	1.7	4	16
Tobramycin	0.12–>64	77.5	0.8	21.7	1	>64
Netilmicin	0.25–>128	56.7	28.3	15.0	8	32
Gentamicin	\leq 0.25–128	53.3	21.7	25.0	4	128
Ciprofloxacin	0.06–128	69.2	2.5	28.3	0.25	32

^a As defined by the CLSI breakpoints. S, susceptible; I, intermediate; R, resistant.

^b No intermediate category has been defined for this antibiotic by the CLSI.

Analysis of β -lactam resistance mechanisms. The β -lactamase content of the selected strains was first analyzed by isoelectrofocusing (pI) (14) and then confirmed by gene sequencing with consensus primers targeting the *tem*, *shv*, and *oxa* genes (1, 4, 30). In addition, the activities of intrinsic β -lactamase AmpC were quantified spectrophotometrically with the chromogenic substrate nitrocefin (14). Resistant isolates exhibiting basal (uninduced) AmpC activities at least fourfold higher than that of wild-type strain PAO1 (20 nmol/min/mg) were considered to be cephalosporinase-derepressed mutants (21). As expected, the susceptibility of all these mutants to β -lactams (except carbapenems) was partially or completely restored in the presence of the AmpC inhibitor cloxacillin at 1,000 $\mu\text{g/ml}$ (data not shown). The production of the porin OprD was assessed for deficiency by RT-PCR targeting the *oprD* gene (9) in those isolates with reduced susceptibility to imipenem (MIC, \geq 8 $\mu\text{g/ml}$; n = 21).

Analysis of aminoglycoside resistance mechanisms. Genes encoding aminoglycoside-modifying enzymes were identified by PCR. Specific sequences of sense and antisense primers were chosen within the nucleotide sequences of the following widely distributed genes in clinical *P. aeruginosa*: *aac(6')*-Ib (5'-TTGCAATGCTGAATGGAGAG-3' and 5'-CGTTTGATCTTGGTGACCT-3'), *ant(2'')*-Ia (5'-GAGCGAAATCTGCCGCTTGG-3' and 5'-CTGTTACAACGACTGGCCGC-3'), and *aac(3)*-Ia (5'-ACCTACTCCCAACATCAGCC-3' and 5'-ATATAGATCTCACTACGCGG-3') (35). Strains showing a moderate resistance to all the aminoglycosides tested, including the enzyme-recalcitrant compounds apramycin and fortimicin, were considered to be harboring at least one nonenzymatic resistance mechanism to these antibiotics (i.e., not involving the production of aminoglycoside-modifying enzymes).

Characterization of gain-of-efflux mutants. The constitutive up-regulation of the efflux pumps MexAB-OprM and MexXY-OprM was assessed in the 120 clinical strains by (i) semiquantitative estimation of proteins MexB, OprM, and MexY on membrane extracts by Western blotting and (ii) RT-PCR determination of the transcriptional levels of genes *mexB* and *mexY*, as reported previously (9, 15, 17). MexXY-OprM-dependent resistance to aminoglycosides and fluoroquinolones was confirmed by overexpressing the plasmid-borne repressor gene *mexZ* in 10 randomly selected strains (plasmid pAZ17 [36]) and by measuring the residual resistance levels of these bacteria subsequent to *mexXY* switch-off (15; data not shown).

QRDR sequencing. A search for mutations in the quinolone resistance-determining regions (QRDR) of genes *gyrA*, *gyrB*, *parC*, and *parE* was performed in all strains for which the ciprofloxacin MIC was at least equal to 1 $\mu\text{g/ml}$, as described previously (14).

RESULTS

Epidemiological data. One hundred twenty bacteremic isolates of *P. aeruginosa* corresponding to 108 genotypically different strains were recovered from 119 patients admitted to six French teaching hospitals between 1999 and 2004. One patient experienced two septicemic episodes with the same strain at a 43-day interval. Ninety-seven of the collected strains were represented by a single isolate, 10 strains by 2 isolates, and 1 strain by 3 clonally related isolates, respectively. No hospital-to-hos-

pital spread of strains was noted during the survey. Concordant with other data in the literature (8), the 30-day mortality rate after the initial bacteremic episode (arbitrarily defined as the date of the first positive blood culture) was quite high (32%) in this series, with 39% of these deaths directly attributable to bacteremia. The infections were associated with severe underlying conditions, such as leukemias (21%), solid tumors (20%), concurrent infectious diseases (12%), or chronic lung diseases (10%).

Antibiotic resistance. The rates of susceptibility of the bacteremic isolates to 11 antipseudomonal antibiotics used in French hospitals are shown in Table 1. Piperacillin-tazobactam, ceftazidime, and amikacin were the most-frequently active agents (\geq 90% susceptible strains). According to the CLSI breakpoints, only 37% (45 of 120) of the isolates were susceptible to all the products tested, with many isolates (63%) exhibiting intermediate or high resistance to at least one antibiotic (up to 9 for three strains) (Fig. 1). From a microbiological viewpoint, as many as 75% (n = 90) of the bacteremic *P. aeruginosa* isolates displayed a significant resistance (fourfold or greater increase in the MIC of a given antibiotic compared with that for wild-type reference strain PAO1) to one or more of the tested antimicrobials (up to 11 for 1 strain) (Fig. 1). To get an insight into the resistance mechanisms prevailing in these strains, we therefore analyzed several determinants known to increase the MICs of β -lactams, aminoglycosides, and/or fluoroquinolones at least fourfold.

Resistance to β -lactams. Analysis of the 81 isolates showing an increased resistance to β -lactams (as microbiologically defined above) revealed a complex situation involving the overexpression of active efflux systems (MexAB-OprM and/or MexXY-OprM), stable up-regulation of the intrinsic cephalosporinase AmpC, the production of various secondary β -lactamases (PSE-1, TEM-2, or OXA-2), and/or defective production of the carbapenem-specific porin OprD (Table 2). No determinants of resistance to β -lactams could be identified in 12 bacteria exhibiting low susceptibility to piperacillin-tazobactam (n = 5; MIC, 8 $\mu\text{g/ml}$ versus 2 $\mu\text{g/ml}$ for PAO1) and/or cefepime (n = 8; MIC, 4 $\mu\text{g/ml}$ versus 1 $\mu\text{g/ml}$ for PAO1). The remaining 69 strains were found to individually express one (n = 37), two (n = 22), or three (n = 10) different mechanisms of β -lactam resistance. These mechanisms were more often

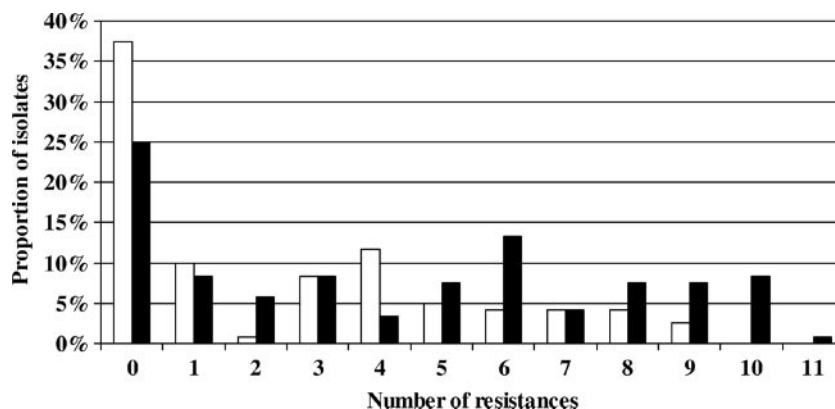


FIG. 1. Multidrug resistance in the bacteremic *P. aeruginosa* isolates. White bars represent the percentage of isolates exhibiting intermediate susceptibility or resistance (CLSI breakpoints) to a given number of antibiotics. Black bars refer to a microbiological definition of resistance (MIC, at least fourfold that of the reference strain PAO1).

intrinsic (i.e., resulting from chromosomal mutations; $n = 58$) than exogenous (i.e., resulting from genetic transfers; $n = 11$). For instance, up-regulation of the β -lactamase AmpC or efflux pump MexXY-OprM occurred in 23 and 43 isolates, respectively. MexAB-OprM overproducing was less prevalent ($n = 13$). As reported elsewhere (21), AmpC derepression alone caused a 4- to 16-fold increase in the MICs of most of the β -lactams tested, except imipenem. In comparison, efflux-based resistance was somewhat lower (two- to eightfold MIC increase) and restricted to compounds such as cefepime (for MexXY-OprM-overproducing mutants), ticarcillin, and aztreonam (for MexAB-OprM-overproducing mutants). No carbapenemase or extended-spectrum β -lactamase was detected in this study. An alteration in the production of porin OprD typically led to a mean eightfold increase in resistance to the carbapenem imipenem. In 17 strains, *oprD* transcripts could

not be detected by RT-PCR, while in 4 others, the expression of the gene was reduced (0.39- to 0.55-fold that of PAO1).

Resistance to aminoglycosides. Half of the isolates (60 of 120; 50%) displayed a minimum fourfold-increased resistance to one or more aminoglycosides (Table 3). RT-PCR and gene complementation experiments showed that a large proportion of these bacteria (43 of 60; 72%) overproduced the efflux system MexXY-OprM. The production of common transferable modifying enzymes, such as ANT(2'')-I, AAC(6')-I, and AAC(3')-I, was detected in 25 strains (42%) that were highly resistant to specific aminoglycosides, among which 19 (76%) overexpressed MexXY-OprM. MexXY-OprM up-regulation increased the MICs of aminoglycosides from two- to fourfold regardless of the simultaneous production of modifying enzymes by the bacteria (Table 3). Concordant with this, the proportion of MexXY-OprM gain-of-efflux mutants was found

TABLE 2. Mechanisms of resistance to β -lactams detected in the bacteremic *P. aeruginosa* isolates ($n = 69$)

Strain(s) and resistance mechanism(s) ^a	No. of isolates (no. <i>oprD</i> negative)	Modal MIC ^b (μ g/ml)					
		FEP	CAZ	ATM	TIC	TZP	IPM ^c
Wild-type reference strain PAO1		1	1	4	16	2	1
Wild-type bacteremic isolates	39	2	1	4	16	4	1
Resistant bacteremic isolates							
XY ⁺	19 (4)	4	2	4	16	4	8
AmpC ⁺ , XY ⁺	11 (5)	16	8	16	128	64	16
OprD ⁻	8 (8)	4	2	4	16	4	8
AmpC ⁺	7 (2)	8	16	16	64	32	8–64
PSE-1, XY ⁺	5 (1)	16	2	8	>256	64	64
AmpC ⁺ , XY ⁺ , PSE-1	1 (0)	16	8	16	>256	128	
PSE-1	2 (0)	16	2–4	8–16	>256	128–256	
AmpC ⁺ , AB ⁺	2 (1)	8	4	32	128	16	8
AB ⁺	6 (0)	8	4	16	128	16	
AB ⁺ , XY ⁺	4 (0)	8	2	16	128	8	
AmpC ⁺ , XY ⁺ , TEM-2	1 (0)	16	32	16	>256	64	
OXA-2	1 (0)	2	2	16	128	8	
AmpC ⁺ , AB ⁺ , XY ⁺	1 (0)	8	16	16	128	64	
XY ⁺ , TEM-2	1 (0)	16	8	8	>256	64	

^a Overproduction of MexXY-OprM (XY⁺) or MexAB-OprM (AB⁺) pump; production of PSE-1, TEM-2, or OXA-2 β -lactamase; stable overexpression of cephalosporinase AmpC (AmpC⁺), and deficiency in OprD (OprD⁻).

^b MICs in the intermediate or resistant categories, according to CLSI breakpoints, are in boldface. FEP, cefepime; CAZ, ceftazidime; ATM, aztreonam; TIC, ticarcillin; TZP, tazocillin/piperacillin; IPM, imipenem.

^c MICs of imipenem are only given for OprD⁻ isolates.

TABLE 3. Mechanisms of resistance to aminoglycosides detected in the bacteremic *P. aeruginosa* isolates (*n* = 60)

Strain(s) and resistance mechanism(s) ^a	No. of isolates	Modal MIC ^b (μg/ml)			
		NET	TOB	AMK	GEN
Wild-type reference strain PAO1		4	0.5	4	2
Wild-type bacteremic isolates	60	4	1	4	4
Resistant bacteremic isolates					
XY ⁺	24	16	2	8	8
Nonenzymatic, MexXY-OprM-independent resistance	11	16	2	8	8
XY ⁺ , ANT(2'')-I	10	16	>64	8	128
XY ⁺ , AAC(6')-I	5	>128	>64	32	128
ANT(2'')-I	3	8	16	4	>128
AAC(6')-I, ANT(2'')-I	2	>128	64	16	128
XY ⁺ , AAC(6')-I, ANT(2'')-I	2	>128	64	16	128
XY ⁺ , AAC(3)-I	1	16	2	8	128
XY ⁺ , AAC(6')-I, AAC(3)-I	1	>128	>64	128	128
AAC(6')-I	1	>128	32	16	128

^a Production of aminoglycoside-modifying enzymes ANT(2'')-I, AAC(6')-I, and AAC(3)-I and overproduction of MexXY-OprM pump (XY⁺).

^b MICs in the intermediate or resistant categories according to CLSI breakpoints are in boldface. NET, netilmicin; TOB, tobramycin; AMK, amikacin; GEN, gentamicin.

to increase along with the resistance levels to aminoglycosides (3, 68, 55, and 100% at amikacin MICs of ≤4, 8, 16, and ≥32 μg/ml, respectively). Of note, no mechanism could be identified in 11 isolates showing a modal fourfold-increased resistance to all the aminoglycosides tested, including the enzyme-recalcitrant compounds apramycin and fortimicin (data not shown).

Resistance to fluoroquinolones. Not less than 61% (73 of 120) of the bacteremic strains exhibited an increased resistance to ciprofloxacin (MIC, ≥0.25 μg/ml versus 0.06 μg/ml for PAO1). Of the 36 strains displaying a low-level resistance to ciprofloxacin (MIC, 0.25 to 1 μg/ml), 9 (25%) and 13 (36%) overproduced the MexAB-OprM or MexXY-OprM efflux pumps, respectively, with three strains overproducing the two pumps simultaneously. The other 37 isolates were nonsusceptible to ciprofloxacin according to the CLSI breakpoint (MIC, ≥2 μg/ml). The canonical Thr-83→Ile substitution in *gyrA* was

found in 35 of these strains, concomitant with a Ser-80→Leu or Ser-80→Trp substitution in *parC* for 27 of them. Isolates with a double GyrA/ParC mutation were more resistant (modal ciprofloxacin MIC, 32 μg/ml) than strains displaying a single GyrA mutation (modal MIC, 16 μg/ml). One resistant isolate (MIC, 32 μg/ml) displayed a single mutation in *parC* associated with MexXY-OprM overproduction, while another (MIC, 8 μg/ml) did not exhibit known resistance mechanisms to fluoroquinolones. No mutation was observed in the QRDRs of *gyrB* and *parE* genes. Interestingly, most of these resistant isolates (29 of 37; 78%) turned out to overexpress MexXY-OprM in a proportion that increased with the levels of resistance to ciprofloxacin (60, 70, 94, and 100% of the isolates with MICs of 4 to 8, 16, 32, and 128 μg/ml, respectively). Finally, none of 73 isolates displayed a resistance profile compatible with efflux systems MexCD-OprJ and MexEF-OprN being overproduced (hypersusceptibility to most β-lactams and/or to aminoglycosides) (28). This finding agrees with other data showing the low prevalence of MexCD-OprJ- and MexEF-OprN-overexpressing mutants among French clinical strains of *P. aeruginosa* (16).

DISCUSSION

Multidrug resistance in clinical isolates of *P. aeruginosa* is an increasing threat in hospitals worldwide (22). To our knowledge, an extensive analysis of the mechanisms of resistance to the three major classes of antipseudomonal compounds (β-lactams, aminoglycosides, and fluoroquinolones) in invasive strains of *P. aeruginosa* has not yet been conducted. This study revealed that a great proportion (75%) of bacteremic strains no longer exhibited a wild-type profile of susceptibility to antimicrobials (MIC, at least fourfold that of the reference strain PAO1). Consistent with this, 64% of the isolates harbored one or several resistance mechanisms (up to six in three strains and seven in one strain) (Fig. 2). The observation that these mechanisms are mostly intrinsic reinforces the notion that *P. aeruginosa* may readily adapt itself to the antibiotic pressure via

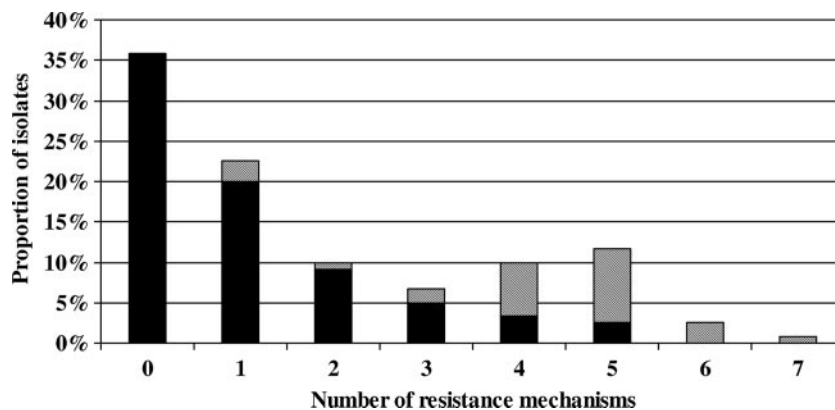


FIG. 2. Accumulation of distinct resistance mechanisms to antibiotics in the 120 bacteremic *P. aeruginosa* isolates. The black bars represent the rates of isolates exhibiting only intrinsic resistance mechanism(s) (overexpression of the AmpC β-lactamase, decreased production of porin OprD, up-regulation of efflux systems MexAB-OprM and/or MexXY-OprM, nonenzymatic resistance to aminoglycosides independent of MexXY-OprM, and mutation[s] in the QRDRs). The hatched bars represent the rates of isolates showing both intrinsic and exogenous (production of transferable β-lactamases and aminoglycoside-modification enzymes) mechanisms of resistance.

chromosomal mutations and does not necessarily require the transfer of foreign DNA (22).

Such an accumulation of independent mechanisms allows individual strains to become resistant to a large range of antibiotics (complementary effects of the mechanisms) and to survive the most-potent drugs (cooperative effects). For example, the simultaneous overexpression of the cephalosporinase AmpC and efflux system MexXY-OprM in the bacteremic isolates (9%) resulted in resistance to many β -lactams, including the stable third-generation cephalosporin β -lactamase cefepime, as well as to fluoroquinolones and aminoglycosides. Up-regulation of MexXY-OprM reinforced the resistance to aminoglycosides conferred by modifying enzymes (Table 3) and the resistance to fluoroquinolones due to mutations in the QRDRs. As previously noted, synergistic interactions between efflux pumps and β -lactamases were less evident (24).

Interestingly, 15 of the 45 isolates susceptible to all the tested antibiotics (as defined by the CLSI breakpoints) demonstrated low levels of resistance to at least one antibiotic. Ten out of these 15 exhibited a low level of resistance to ciprofloxacin (MIC, 0.25 to 0.5 μ g/ml). According to pharmacokinetic/pharmacodynamic studies, a moderate resistance to fluoroquinolones is associated with a dramatic decrease in the target attainment rates that predict therapeutic outcome (10, 18). In some strains, the mechanisms responsible for the low-level resistance to fluoroquinolones ($n = 14$) and to aminoglycosides ($n = 11$) could not be characterized. Alteration of the lipopolysaccharide, production of Qnr proteins, and/or decreased uptake of aminoglycosides in these bacteria are currently being investigated (26, 34). Whether these low-level-resistance mechanisms may favor the emergence of secondary mutants with stronger resistance to fluoroquinolones or aminoglycosides needs to be confirmed (3).

Recently, cellular and animal models of infection have suggested that, when overexpressed, Mex pumps might negatively impact the virulence in *P. aeruginosa* (11, 13, 20, 29, 32). In apparent contrast with these studies, our data show that MexAB-OprM or MexXY-OprM are frequently overproduced in bacteremic strains (11% and 36% of the isolates, respectively). A trivial explanation could be that, in the clinical setting, gain-of-efflux mutants may recover their fitness or virulence by compensatory mutations. Supporting this notion, it has been demonstrated that, while the acquisition of resistance determinants in pathogenic bacteria usually leads to a decreased virulence (5), secondary mutations may easily restore the initial bacterial fitness (5, 6). Of interest, the crude and attributable mortalities in this series were not correlated with the number and nature of the resistance mechanisms accumulated by the bacteremic isolates (data not shown).

In conclusion, this study demonstrates that *P. aeruginosa* strains with multiple drug resistance mechanisms remain invasive and can cause severe infections.

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